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Expression of the Marker(X)(q28) in Lymphoblastoid Cell Lines

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SUMMARY

The marker(X)(q28) chromosome associated with one type of X-linked mental retardation has been demonstrated in lymphoblastoid cell lines established from affected individuals. The mar(x) can reliably and repeatedly be seen by the addition of FUdR to the cultures for 24 hrs prior to harvest. This simple technique provides an excellent in vitro experimental test system for investigation of the mar(X).

INTRODUCTION

The recent demonstration that a common form of X-linked mental retardation is associated with a cytologically detectable marker on the X chromosome of lymphocytes of affected males and a proportion of carrier females is of considerable theoretical and practical importance [1–3].

The marker(X)(q28) has been reliably and repeatedly demonstrated in lymphocytes in short-term culture and then only when the cells are grown under special culture conditions. It was first shown by Sutherland [4] that the mar(X) was seen only when cells from affected individuals were cultured in medium 199, which contains relatively low concentrations of folic acid, thymidine, and hypoxanthine, and that the expression of the mar(X) was enhanced at low serum concentrations and relatively high pH. Glover [5] demonstrated the mar(X) by the addition of FUdR to short-term lymphocyte cultures grown in medium containing folic acid,

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suggesting that the demonstration of the mar(X) depended on thymidine depletion rather than on folic-acid deprivation per se.

Even under optimum conditions, the mar(X) is seen in only some of the lymphocytes from affected males and is not seen at all in lymphocytes from many obligate carrier females, especially those who are over age 30 [6]. Glover [5] and Tommerup et al. [6] reported preliminary observations suggesting that the mar(X) was demonstrable in fibroblasts by the addition of FUdR. However, it is not yet known whether this technique is reliable.

The marker(X)(q28) is, to our knowledge, the first cytogenetically demonstrable aberration reported in association with a Mendelian gene. Until now, attempts to understand the mechanism underlying this phenomenon have been hampered by the absence of an in vitro experimental test system. Here we report the development of such a system, namely, lymphoblastoid cell lines, and present the results of our first experiments using such lines.

MATERIALS AND METHODS

Human lymphoblastoid cell lines were initiated by Epstein-Barr virus transformation of peripheral blood lymphocytes according to the following technique (modified from Sly et al. [7]). Heparinized blood was diluted with an equal volume of RPMI 1640 (Gibco, Grand Island, N.Y.) containing 1% penicillin and streptomycin. The mixture was then gently layered over a 55% (v/v) Percoll (Pharmacia, Uppsala, Sweden) in phosphate buffer solution in a 50-ml centrifuge tube at a 3:1 ratio. The tube was centrifuged at 2,500 rpm for 20–30 min. The layer that appeared at the plasma-Percoll interface contained lymphocytes and was carefully removed and washed twice with RPMI 1640 medium (by sedimentation at 1,000–1,200 rpm for 10 min). The washed lymphocytes were then resuspended at a final concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 15% fetal bovine serum (Gibco), 4 mM glutamine, and 20 mM Hepes buffer (pH 7.2). To the culture were added 10% (v/v) Epstein-Barr virus prepared from Marmoset lymphoid line B 95-8 [8] and 2 μg/ml cyclosporin A [9]. The culture was then incubated at 37°C in an atmosphere of 95% air and 5% CO_2 and fed every 5–7 days with 1–2 ml of fresh medium. Transformation was evident by the appearance of multiple clumps of growing cells. Fully established lymphoblastoid cells were either frozen for storage and subsequent propagation or maintained in culture in RPMI 1640 supplemented with 15% fetal bovine serum at a density between $2–10 \times 10^5$ cells/ml. All cells were tested for mycoplasma by both bioassay and DNA staining methods [10].

Four lines were initiated at the Massachusetts General Hospital and sent to Hawaii. MGL8B2 (L8) was a twice-cloned line established from a normal 27-year-old male. MGL 29 (L29) was established from a mentally retarded 22-year-old male who was shown to have the marker in 27% of his peripheral lymphocytes. MGL 42 (L42) and MGL 43 (L43) were from two sisters of an affected male. L42 was from a normal 47-year-old female who did not show the marker in peripheral lymphocytes while L43 was from a mentally retarded 40-year-old who showed the marker(X) in 7% of her peripheral lymphocytes.

In Hawaii, the cultures were maintained in D medium (Gibco, cat. #78-5470) modified by the addition of pyruvate, glucose, and glutamine and supplemented with 20% fetal bovine serum. When experiments to look for the mar(X) were initiated, the cells were resuspended in 10 ml of either D or M medium (modified F10 without folic acid, thymidine, or hypoxanthine) supplemented with 5% fetal bovine serum. Cytogenetic preparations were made by standard techniques, and the cells stained in lactic-acetic-orcein and scored blindly by direct microscopic examination.

TABLE 1

RESULTS OF EXPERIMENTS WITH LYMPHOBLASTOID CELL LINES

EXPERIMENT NO.	CELL LINE	MEDIUM	SERUM (%)	INITIAL CELL COUNT (CELLS/ML)	−FUdR*	+FUdR†		MITOTIC INDEX‡	TOTAL CELLS SCORED§	Mar(X)	
					Time (hrs)	Time (hrs)	Concentration (M)			No.	%
1 A	L8	M	5	...	8	24	10^{-7}	2	18	0	0
B	L29	M	5	...	8	24	10^{-7}	1	85	39	46
C	L8	M	5	...	8	48	10^{-7}	3	85	0	0
D	L29	M	5	...	8	48	10^{-7}	2	88	44	50
2 A	L29	M	5	...	18	24	0.5×10^{-7}	3	50	27	54
B	L29	M	5	...	18	24	10^{-7}	1	38	24	63
C	L29	M	5	...	18	24	2×10^{-7}	1	52	22	42
D	L29	M	5	...	18	48	0.5×10^{-7}	3	75	18	24
E	L29	M	5	...	18	48	10^{-7}	4	75	17	23
F	L29	M	5	...	18	48	2×10^{-7}	3	55	20	36
3 A	L29	M	5	...	24	24	10^{-7}	2	60	25	42
B	L29	M	5	...	48	...	...	4	66	1	1
C	L29	M	5	...	24	48	10^{-7}	1	14	5	36
D	L29	M	5	...	72	...	...	2	42	0	0
4 A	L29	M	5	3.5×10^{5}	...	24	10^{-7}	2	93	43	46
B	L29	D	5	3.5×10^{5}	...	24	10^{-7}	1	103	44	43

TABLE 1 (*Continued*)

Experiment No.	Cell Line	Medium	Serum (%)	Initial Cell Count (cells/ml)	−FUdR*	+FUdR†		Mitotic Index‡	Total Cells Scored§	Mar(X)	
					Time (hrs)	Time (hrs)	Concentration (M)			No.	%
5 A	L29	M	5	Very high	...	48	10^{-7}	4	75	2	3
B	L29	M	15	Very high	...	48	10^{-7}	5	75	5	7
C	L29	D	15	Very high	...	48	10^{-7}	4	75	3	4
6 A	L29	M	5	1.5×10^5	...	24	10^{-7}	1	96	58	60
B	L29	M	5	5×10^5	...	24	10^{-7}	2	120	50	42
C	L29	M	5	2×10^6	...	24	10^{-7}	3	75	6	8
D	L29	M	5	3×10^6	...	24	10^{-7}	2	75	4	5
7 A	L29	M	5	6×10^4	...	24	10^{-7}	1	44	13	29
B	L29	M	5	1.5×10^5	...	24	10^{-7}	1	57	29	51
C	L29	M	5	3×10^5	...	24	10^{-7}	2	53	22	41
D	L29	M	5	6×10^5	...	24	10^{-7}	2	59	30	51
E	L29	M	5	4×10^6	...	24	10^{-7}	2	50	21	42
8 A	L42	M	5	3×10^5	...	24	10^{-7}	1	30	0	0
B	L42	M	5	3×10^5	...	24	10^{-7}	1	51	0	0
C	L43	M	5	3×10^5	...	24	10^{-7}	2	67	9	13
D	L43	M	5	3×10^5	...	24	10^{-7}	2	67	9	13
9 A	L43	M	5	2×10^5	...	24	10^{-7}	3	92	12	13
B	L43	M	5	3×10^5	...	24	10^{-7}	2	69	10	14

* −FUdR time at beginning of each experiment before FUdR added.
† +FUdR time after addition of FUdR to culture.
‡ Scored subjectively: 1 = very low; 2 = low; 3 = average; 4 = high; 5 = very high.
§ Only diploid cells included.

RESULTS

The results of nine experiments done with the lymphoblastoid cell lines are given in table 1. As can be seen, the mar(X) was demonstrable in a relatively high proportion of cells from cultures L29 and L43 but not at all in the control cultures. The presence of FUdR (experiment 3), but not the absence of folic acid (experiment 4), seems necessary to demonstrate the mar(X) in this system. It is surprising that the use of M medium without FUdR failed to show the mar(X) as this is the technique that we routinely used to demonstrate the mar(X) in short-term lymphocyte cultures. Over the range that we tested, neither the concentration of FUdR nor the time of exposure of the cells to FUdR appeared critical for demonstration of the mar(X), although there was some suggestion (experiment 2) that longer exposure was associated with a reduction in the proportion of cells showing the mar(X). Initially the cells were "conditioned" to M medium and 5% serum for 8–24 hrs prior to the addition of FUdR, but this proved unnecessary and was omitted in later experiments.

Experiment 5, initiated to test serum concentration, showed an unexpectedly low proportion of cells with the mar(X) even under conditions in which it had been seen in high frequency previously. All cultures in experiment 5 contained a very large number of cells and had a high mitotic index. Therefore, experiments 6 and 7 were designed to test the effect of cell density on expression of the mar(X). While experiment 6 demonstrated a marked lowering of the proportion of mar(X) positive cells with increasing cell density, this was not evident in experiment 7. Thus if cell density has an effect on the expression of the mar(X), it appears to be in conjunction with some other, as yet unknown, factor.

DISCUSSION

While we do not understand the mechanism responsible for mar(X) expression, the demonstration that the mar(X) is expressed in appropriately treated cells of lymphoblastoid cell lines established from affected individuals will, by providing almost ideal experimental material, greatly facilitate attempts to understand this phenomenon. Our preliminary results have shown, first, that lymphoblastoid cell lines are easily established from individuals with the mar(X) type of X-linked mental retardation, and, second, that the mar(X) can reliably and repeatedly be demonstrated by the simple expedient of adding FUdR for 24 hrs to cells growing in medium in which thymidine and serum concentrations are reduced.

In our preliminary observations, we are impressed by the constancy in the proportion of cells showing the mar(X) in a given cell line. Thus in L29, the affected male, we virtually never observed significantly more than 50% of the cells to have the mar(X), while in four separate tests on L43, the affected female, we found only about 13% of the cells to have the mar(X). These proportions of mar(X) positive cells are about double the 27% and 7%, respectively, seen in the peripheral blood lymphocytes from these patients. While our failure to demonstrate the mar(X) in more cells may be the result of suboptimal conditions, it may be that the maximum number of mar(X) positive cells in lymphoblastoid cell lines

is related to the number seen in peripheral blood leukocytes and differs among individual patients.

If the latter explanation is valid and each patient has a characteristic proportion of mar(X) positive cells, it is interesting to note that, in observations published to date, the proportion of mar(X) positive cells is usually considerably less than 50% and rarely, if ever, significantly above 50%. If it is substantiated that only one-half of the cells can show the mar(X), this should provide an important clue to the nature of the phenomenon. Further observations on lymphoblastoid cell lines from more patients and on clones established from the same cell line will undoubtedly clarify many of the issues surrounding the mar(X).

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